ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Influence of Chitooligosacharides with Different Degrees of Acetylation On the Activity of Protective Proteins in Wheat Leaves During Infection with Septoria Pathogen.

LG Yarullina[1, 2]*, RI Kasimova[1], RI Ibragimov[2], VO Tsvetkov[2], AR Akhatova[1, 3], LM Yarullina[2], and IV Maksimov[1]

[1] Institute of Biochemistry and Genetics USC RAS, 450054, Russia, City of Ufa, Oktyabrya av., 71
[2] Bashkir State University, 450076, Russia, City of Ufa, Validi st., 32.
[3] Bashkir State Medicine University, 450000, Russia, City of Ufa, Lenina st., 3.

ABSTRACT

By using RT-PCR there was studied the effect of chitooligosacharides (COS) with different degrees of acetylation (DA) on gene expression of protective proteins (oxalate oxidase, peroxidase, proteinase inhibitors) in leaves of wheat (*Triticum aestivum L.*), infected with pathogen *Septoria* (*Septoria nodorum* Berk.). The development of the fungus in control and pretreated by chitooligosacharides wheat leaves was estimated. It was shown that chitooligosacharides with the DA 65% had the maximum efficiency in enhancing of gene expression of peroxidase and oxalate oxidase, and in suppressing the growth of the pathogen in plant tissues. Increasing the gene expression of proteinase inhibitors, by contrast, was more significant under the influence of chitooligosacharides with the DA 30%.

Keywords: Triticum aestivum, oxalate oxidase, peroxidase, proteinase inhibitors, chitooligosacharides acetylation, Septoria nodorum.

***Corresponding author**

ISSN: 0975-8585

INTRODUCTION

An important trend in plant protection is the search for ecologically safe preparations, which is based on the induction of natural defence mechanisms of plants. In recent years, significant progress has been made in the study of formation mechanisms of defense reactions of plants against phytopathogens. Primarily, this is due to the discovery and practical use of elicitors inducing the launch of local and systemic defense responses of the plant organism.

It is shown that effective elicitors of defense reactions of plants are derivatives of chitin [1]. Using DNA microarrays in *Arabidopsis thaliana* identified 60 genes that are responsive to the addition of chitin [2, 3]. Among them rapid (10-30 min) and high expression were the property of genes responsible for the synthesis of regulatory proteins involved in transcription and transmission of information [3]. A very important property of preparations on the basis of chitin and chitosan from the point of view of practical crop production is their ability to regulate plant's growth at low concentrations [4, 5].

It is believed that a degree of acetylation of the biopolymer has a role in the specificity of biological activity of derivatives of chitin [6, 7]. This is confirmed by the fact that at pathogens were detected enzymes that alter the degree of its acetylation [8, 9].

To develop the technology of using drugs on the basis of chitin itis necessary to obtain an information about the relationship of biological activity with the structure of the biopolymer, otherwise we can get the suppression of a protective response instead of its induction [10]. One of the mechanisms of protective action of low molecular weight chitin derivatives – chitooligosaccharides (COS), is the activation of genes that have a part at the synthesis of pathogen-induced proteins [11, 12]. Pathogen-induced proteins, which are characterized by a wide spectrum of physiological actions, include peroxidase (PO) [13], oxalate-oxidase (OxO) [14], inhibitors of proteinases (PI) of plants [15].

To clarify the role of the degree of acetylation of chitin derivatives in the manifestation of their biological activity, in the present work we investigated the effect of chitooligosaccharides with DA 30 and 65% on the growth of the fungus *S. nodorum* and OxO, PO, PIgenes expression in the leaves of wheat.

EXPERIMENTAL PART

As objects of study were used the seedlings of wheat *T. aestivum* L., varietiyZhnitsa. Fully expanded leaves of 7-day-old wheat seedlings were cut, placed in a moist chamber on filter paper, the sections were covered with cotton wool soaked in a solution of benzimidazole (40 mg/l). Segments of the leaves were inoculated by suspension of pycnospores of *S. nodorum* Berk. (10^6 spores/ml). Infected leaves were maintained at room temperature in the dark for 24 hours and then returned into illuminated conditionswith photoperiod 16 h/day [16]. The symptoms of *Septoria* leaf spot were recorded for 14 days after inoculation of the leaves. Water-soluble chitooligosaccharides with degree of acetylation of 30% and 65%, a molecular weight of 5-7 kDa at a concentration of 1mg/l was used for the soaking of wheat seeds (3 hours). The chitooligosaccharides with 60% ofDA and 30% of DA were acquired in "Sonat" firm, Moscow. After 24, 48 and 72 h after inoculation evaluated the level of PO, OxO, PIgenes expression and activity of proteins coded by them.

Plant material was weighed and triturated in a porcelain mortar with the addition of 0.05M Na-phosphate buffer (PB), pH 6.2 at a ratio of 1 to 5 (1 g of plant material per 5 ml of PB). For isolation of cytoplasmic protein fraction, samples were extracted for 30 min at 4ºC and centrifuged at 15000 rpm, the supernatant was collected. To determine the peroxidase activity, the supernatant was diluted in 30-50 times in PB, pH 6.2 (10 µl of sample per 290 µl of buffer),and placed into the wells of flat-bottomed plate for immunoassay ("Nunc", USA) in the following order: 75 µl of sample, 25 µl of 0.08% of o-phenylenediamine (OPD) ("Reahim", Russia), 25 µl 0.016% H_2O_2. The enzymatic reaction was stopped by 50 µl of 4M sulfuric acid. The optical density was measured on the Benchmark Microplate Reader ("BioRad", USA) for enzyme-linked immunosorbent assay at 490 nm.

Activity of oxalate oxidase in the studied fractions was determined by microtechnique [17] in plates for enzyme-linked immunosorbent assay. Reaction medium contained 100 µl of 0.05 M succinate buffer (pH

3.8), 30 µl of 0.025 M oxalic acid ("Reahim", Russia), 30 µl of enzymatic extract (50-100 µg of protein) and 50 µl of color reagent. To obtain a color reagent we used 0.05 M phosphate buffer (pH 7.0), 0.05% o-phenylenediamine ("Reahim", Russia) and horseradish peroxidase ("Dia-M", Russia) (1 mg/ml). The plate was scanned at a wavelength of 492 nm on a photometer ("Titertek Uniskan", UK).

The activity of inhibitors of proteinases was determined by the method of Hoffmann-Visbly with some modifications [18]. To 0.5 ml of Tris-HCl buffer was added 1.0 ml of the extract and 0.5 ml of enzyme. Then was added 1.0 ml of a solution of Nα-benzoyl–DL-arginine-4-nitroanilide HCl ("Serva", USA), then incubated in a water bath for 10 minutes at 37 °C, then for termination of the reaction was added 0.5 ml of 30% acetic acid ("Khimreaktivsnab", Russia). As a control used a solution consisting of 0.5 ml of Tris-HCl buffer, 1.0 ml of distilled water, 1.0 ml of extract, 0.5 ml of 30% acetic acid and 0.5 ml of enzyme solution. In parallel experiments in which we determined the activity of proteinases, instead of the test solution was added water. The optical absorption of the resulting solution was determined on photometer Labsystems Uniskan ("Labsystems", Finland) at a wavelength of 405 nm.

The activity of the enzyme with and without inhibitors was determined by the formula:

$$A = (E_E – E_X)\cdot V / t \cdot V_1 \cdot 0.00955,$$

where:
A – active enzymes (mIU/ml),
E_E – extinction of proteinase,
E_X– extinction of experienced solution,
V – volume of the incubation mixture,
V_1– volume of enzyme solution (ml),
t – time of proteolysis (min).

Enzyme activity was expressed in inhibitory units (IU). At standard conditions as a unit of inhibitor activity was taking such amount which is necessary to suppress the unit of enzyme activity by 100%.

DNA from plants was isolated using phenol-detergent method. To obtain cDNAs based on an mRNA of the studied samples, the reaction of reverse transcription was carried out using M-MuLV reverse transcriptase ("Fermentas", Lithuania), according to the protocol of the supplier. The concentration of DNA and RNA were measured as A_{260}/A_{280}at spectrophotometer Smart Spec™ Plus (Bio-Rad, USA), after dissolving the samples in Tris-EDTA buffer. Polymerase-chain-reaction (RT-PCR) was performed in the amplifier type TP4-PCR-01"Tertsic" ("DNA-technology", Russia). After amplification, the DNA fragments were fractioned by electrophoresis in 1-2% agarose gel or in the PAGE (7%) in the electrophoretic chamber S2 ("Helicon", Russia). As a positive control used the PCR of the gene encoding constitutive expressed tubulin. Primers to the gene of *Triticum aestivum* (bread wheat) oxalate oxidase precursor (AJ556991.1) F 5'-atg-act-tcc-tct-tct-cgt-cca-ag-3'; R 5'-gga-gct-gaa-gag-tgt-caa-tgg-3' flanks a portion of a gene of oxalate oxidase of 410 BP. Primers to the gene of anionic peroxidase *T. aestivum* F 5'-ttc-gac-aag-cag-tac-tac-cac-aa-3'; R 5'-ccg-aag-tcc-gag-aag-aac-tg-3' are selected on the basis of a gene of wheat (Peroxibase TC 151917), apmlifier of size 157 BP. Primers to the gene of inhibitors of *T. aestivum*proteinases (EU 293132.1) (F 5'-ggg-ccc-tgc-aag-aag-tac-tg-3'; R 5'-aca-cgc-ata-ggc-acg-atg-ac-3', product size is 106 BP. Primers to house-keeping gene β-tubulin DQ 435668.1:Tub. (F) 5'-cac-aca-gca-gat-gtg-gga-ct-3'; R 5'-gtg-gag-ttg-cca-atg-aag-at-3' flanks a stretch of 113 BP.

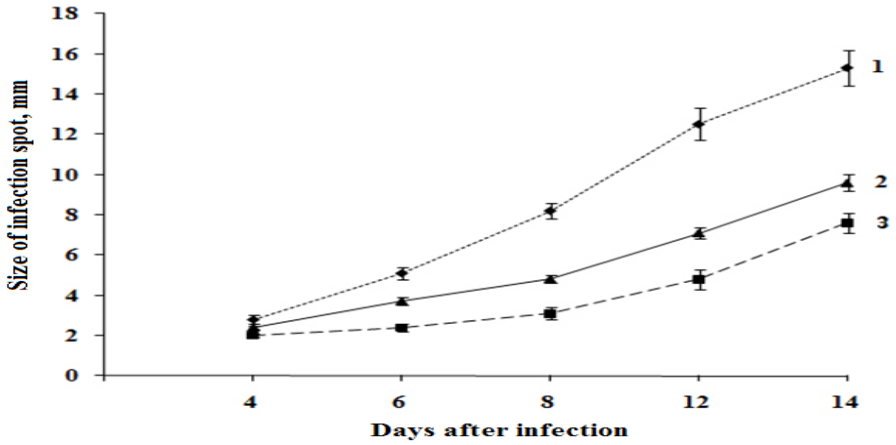
Statistical processing of results was performed using the computer software StatSoft Statistica 6.0. Computer analysis of the amino acid and nucleotide sequences was performed using the software package Lasergene ("DNASTAR, Inc.", USA). The experiments were carried out in 3 biological and 3 chemical replications. In this article we present the photos of PAGE with the best separation of the samples. Quantitative data were processed statistically; the figures show the standard deviation.

RESULTS AND DISCUSSION

The causative agent of glume blotch of *S. nodorum* wheat is parasitic fungi with the hemibiotrophic type of food, i.e. it begins its development in plant tissue as a biotrophic pathogen (living tissue), and ends as necrotroph (dead tissues). Monitoring growth of the pathogen *Septoria* on the epidermis of untreated

(control) and treated by COS leaves of wheat have revealed differences in the degree of development of the fungus (Fig. 1). So on the control wheat leaves after 24 h after infection appeared weak chlorosis and necrosis in the places near thespores. Signs of development of *Septoria* leaf blotch on leaves, preprocessed COS with DA 30% and 65%, were seen after 48 and 72 h after infection, respectively, in the form of a mild discoloring of the leaves. Obtained in these experiments data showed that there is delayed pathogenic growth and development of the fungusunder the influence of COS, especially in the case with the use of COS with DA 65% (Fig. 1).

Fig 1: The effect of chitooligosaccharides with different degree of acetylation on the development of symptoms of Septoria leaf blotch on leaves of wheat, variety Zhnitsa.

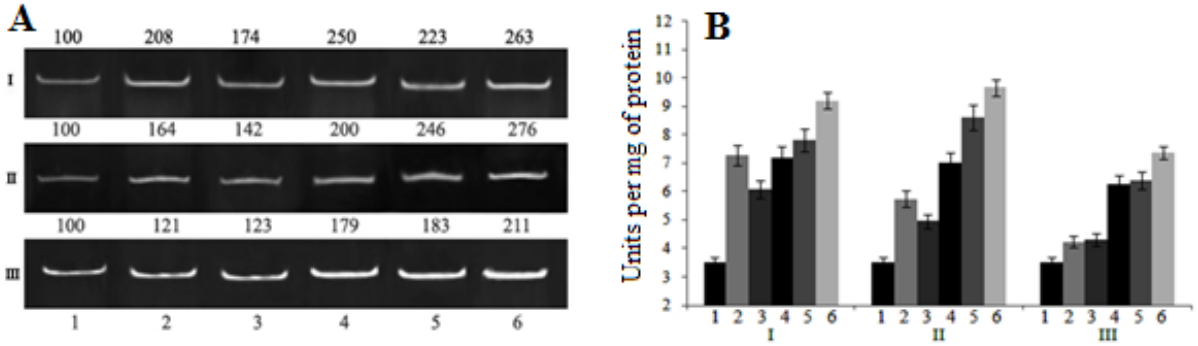


1 – control; 2 – COS, DA 30%; 3 – COS, DA 65%.

As we know, the early response of plants to pathogens is the formation of reactive oxygen species, including hydrogen peroxide [19]. It is shown that the H_2O_2 molecule is formed in plants with the participation of the enzyme oxalate oxidase [20]. Many pathogens of agricultural crops, including the fungus *S. nodorum*, secrete oxalic acid, a pathogenicity factor, in plant tissue [21]. The effectiveness of the protective response of plants to the penetration of the pathogen depend largely on the production of oxalate oxidase (OxO)in plants.

Studies have shown that in response to infection by the pathogen *Septoria*in 24 hours after inoculation of *S. nodorum* the amplification of OxO gene expression(Fig. 2 A - 2) and activity of the enzyme (Fig. 2 B – 2) increases. Subsequently (after 48 and 72 h after inoculation) OxO gene expression (Fig. 2 A - II, III, 2) and, respectively, the activity of the enzyme (Fig. 2 B - II, III, 2) reduced.

Fig 2: Gene of oxalate oxydaseAJ556991.1 expression (A) and enzyme activity (B) in leaves of wheat, variety Zhnitsa, when processing COS with different degree of acetylation and S. nodorum infection.



1 – control, 2 – S. nodorum, 3 – COS, CA 30%, 4 – COS, SA 30% + S. nodorum, 5 – COS, SA 65%, 6 – COS, SA 65% + S. nodorum.
I – 24 hours, II – 48 hours, III – 72 hours.
The numbers above the photos of profiles submitted to gene expression level of an oxalate oxidase in %.

ISSN: 0975-8585

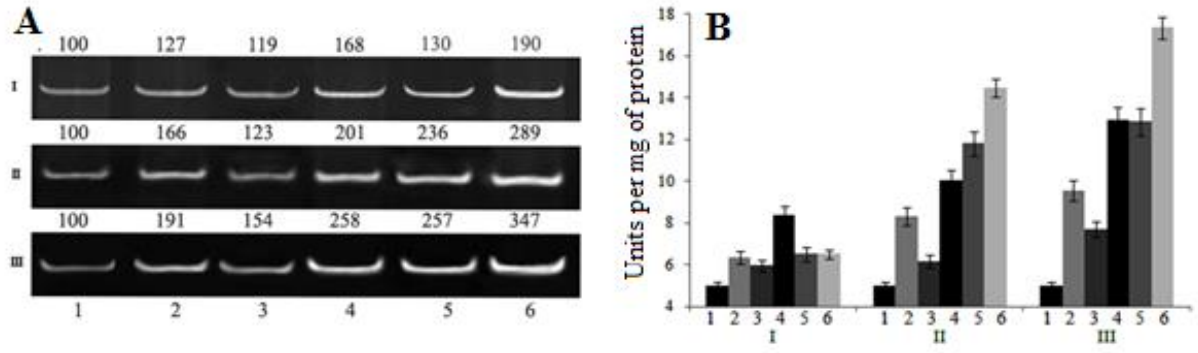
COS depending on the DA showed different catalytic effect on the expression of an OxO gene, both in uninfected and infected leaves (Fig. 2 (A)). Under the influence of COS with DA 65% the expression level of the OxO gene and enzyme activity exceeded the same parameter of infected wheat leaves treated COS with DA 30%, throughout the experiment (Fig. 2 A, B – 4, 6). Moreover, the maximum difference in the induction of OxO gene expression and activity of the enzyme between COS with DA 65% and DA 30% were most clearly in uninfected plants (Fig. 2 A, B – 3, 5).

Increased activity of oxalate oxidase and increased production of hydrogen peroxide during processing of COS with DA 65% were shown during infection of wheat calluses with the causative agent of covered smut [22]. It is now known that reactive oxygen species, including hydrogen peroxide, are an important component of responses to the introduction of pathogens. Protective effect of H_2O_2 caused both by a direct biocidal effect on the pathogen and its participation in signaling systems in plant cells.

In connection with the opening of signal and the protective role of reactive oxygen species, much attention is paid to oxidoreductase regulating their levels in the cell [19, 23]. Among them of special interest is peroxidase, the activity of which correlates with the development of plant resistance to pathogens [24]. It is shown that anionic peroxidase (PO) involved in lignin synthesis, limiting penetration of infectious structures of the fungus into the tissue of a plant [25].

Studies have shown that in response to infection by *S. nodorum* the amplification of POgene expression (Fig. 3 A - 2) and the activity of the enzyme (Fig. 3 B - 2)increases. Moreover, COS with DA 30% had a slight stimulatory effect on the expression of the POgene in uninfected wheat leaves (Fig. 2 - 3). However, when infected with *S. nodorum*, stimulating effect ofCOS with DA 30% markedly increased (Fig. 3 - 4), which was positively reflected on the activity of the enzyme (Fig. 3 B - 4). So after 48 h after inoculation, anionic peroxidase gene expression level was higher than the control variant 2 times, and after 72 h after inoculation 2.5 times (Fig. 3A - 4).

Fig 3: The anionic peroxidase gene TC 151917 expression (A) and enzyme activity (B) in leaves of wheat, variety Zhnitsa, when processing COS with different degree of acetylation and S. nodorum infection.



1 – control, 2 – S. nodorum, 3 – COS, DA 30%, 4 – COS, DA 30% + S. nodorum, 5 – COS, DA 65%, 6 – COS, DA 65% + S. nodorum.

I – 24 hours, II – 48 hours, III – 72 hours.

The numbers above the photos of profiles presents the level of gene expression of an anionic peroxidase in %.

Inducing effect of chitooligosaccharides with DA 65% on the expression of the POgene proved more significant and long lasting both in uninfected (Fig. 3 - 5) and infected leaves (Fig. 3A - 6). In 24 h after inoculation of wheat treated COS with DA 65%, the level of POgene expression was 2.6 times higher than the control variant and remained elevated for 72 h (Fig. 3 A - 6), which could affect the activity of the enzyme (Fig.3 B - 6). A similar effect of COS with DA 65% on activation of peroxidase was detected during infection of wheat plants by root rot pathogen *Bipolaris sorokiniana* [26].

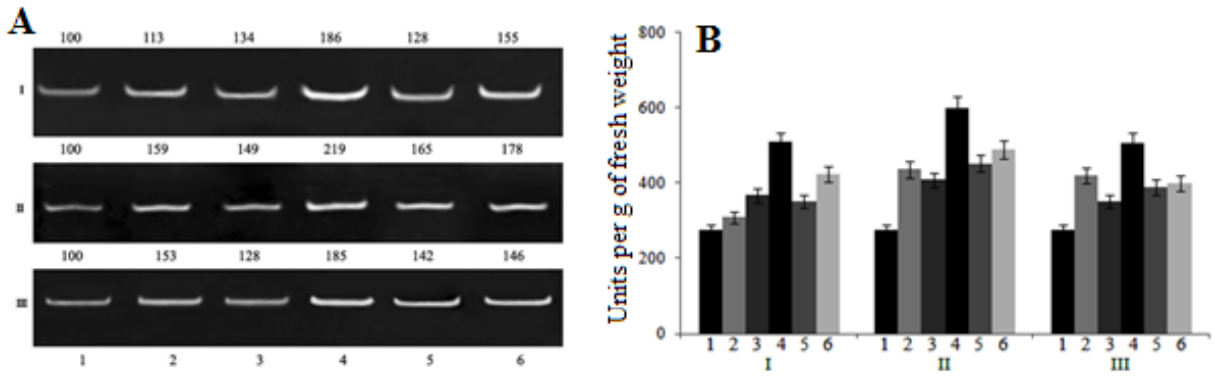
Thus, COS with DA 65% have a more significant inducing effect on the expression of OXO and POgenes compared to COS with DA 30%. Perhaps a higher sensitivity of OxO and POgenes to COS with DA 65% is due to the fact that these enzymes are involved in the regulation of H_2O_2 level at the early stages of the infection

process. OxO oxidizes oxalic acid and oxalates with synthesis of hydrogen peroxide, which a peroxidase uses in reactions of lignification. Considering that chitin is a component of cell walls of fungi, there are chitinous fragments with a high degree of acetylation formedin the process of its destruction by chitinaseon the initial stages of pathogenesis, then they are involved in the regulation of activity of enzymes of the metabolism of ROS. Such a mechanism of the induction of a protective response by COS could be formed in the process of co-evolution of the system "plant – fungal pathogen".

Essential components of pathogenic microorganisms infection in plants are their hydrolytic enzymes. In response to aggressive action of proteinases of the pathogen, protein inhibitors synthesis is induced in the plant, it can be directed on suppression of activity of these enzymes [27, 28].

As can be seen from Fig. 4, in response to inoculation of the pathogen *Septoria* there is an increasing of proteinases inhibitors gene expression and increasing of activity of the protein product. Interestingly, in relation to the activation of inhibitors of proteinases the maximum efficiency was provided by COS with DA 30%. So, after 48 h after infection observed increased expression of proteinases inhibitors genesin 2 times (Fig. 4 A - 4), which is accompanied by increased activity of the enzyme (Fig. 4 B - 4).

Fig 4: Gene expression of inhibitors of proteinases EU 293132.1 (A) and enzyme activity (B) in leaves of wheat, variety Zhnitsa, when processing COS with different degree of acetylation and S. nodorum infection.



1 – control, 2 - S. nodorum, 3 – COS, DA 30%, 4 – COS, DA 30% + S. nodorum, 5 - COS, DA 65%, 6 – COS, DA 65% + S. nodorum.
I – 24 hours, II – 48 hours, III – 72 hours.
The numbers above the photos of profiles presents the level of gene expression of inhibitor of proteinases in %.

The increased content of protease inhibitors in the plant occurs, as a rule, not by increasing the concentration of constitutive compounds, and due to the synthesis of new forms of inhibitors [29, 30]. Probably proteinases inhibitors genes is more sensitive to COS with DA 30%,which can be used for the induction of their activity.

CONCLUSION

Thus, the preparation of chitooligosaccharides can improve the resistance of wheat plants, firstly, by increasing oxalate oxidase gene expression, contributing to the accumulation of hydrogen peroxide in the places of infection, secondly, strengthening the processes of lignification as a result of activation of anionic peroxidase [31] and, thirdly, inducing in plant tissues the activity of inhibitors of proteinases, which contributes to reducing the damaging effects of the pathogen *Septoria*. Moreover, the inducing effect on protective proteins genes expression in wheat plants when infected with *S. nodorum* depends on the degree of acetylation of chitooligosaccharides.

REFERENCES

[1] Oseretskovskaya OL, Vasyukova NI, PaninaYS, Chalenko GI.Plant Physiology 2006; 4: 546-553.*(in Russian)*

[2] Ramonell KM, Zhang B, Ewing R. Mol. Plant Pathol 2002; 3: 301-311.
[3] Zhang B, Ramonell K, Somerville S, Stacey G. Mol. Plant Pathogen Interaction 2002; 15: 963-970.
[4] Petrukhina MT, Maksimov VI, Luntsevich VG, Bordak MN. Proc. dokl. fifth Conf. "New perspectives in the study of chitin and chitosan" 1999; 98-100. *(in Russian)*
[5] Tyuterev SL. The scientific basis of induced disease resistance of plants. S.-Pb, LLC "Innovation center of plant protection" of VIZR, 2002, pp. 328*(in Russian)*
[6] Falcon AB, Cabrera JC, Costales D, Ramirez MA, Cabrera G, Toledo V, Martinez-Tellez MA. World J. Microbiol. Biotechnol 2008; 24: 103-112.
[7] Santos A, Guedarri N, Tromboto S, Moerschbacher BM. Biomacromolecules 2008; 9: 3411-3415.
[8] Tsigos L, Martinou A, Kafetzopoulos D, Bouriotis V.Trends Biotechnolog 2000; 7: 305-312.
[9] Blair DE, Hekmat O, Schuttelkopf AW, Sheresta B, Tokuyasu K, Withers SG, Van Aalten DMF. Biochemistry 2006; 45: 9416-9426.
[10] Oseretskovskaya OL, Vasyukova NI. Applied biochemistry and mikrobiol 2002; 3: 322-325.*(in Russian)*
[11] OseretskovskayaOL, Romenskaya IG. Plant Physiol 1996; 43: 743-752.*(in Russian)*
[12] Perekhod EA, Chalenko GI, Gerasimov NG. Reports of Academy of Sciences 1997; 355: 120-122. *(in Russian)*
[13] Malinovskiy VI. Phys modern biology 2009; 129: 1-9. *(in Russian)*
[14] Yarullina LG, Ibragimov RI. Cellular mechanisms of plant resistance to fungal pathogens. Ufa, Gilem, 2006, 232 p.*(in Russian)*
[15] Mosolov VV, Valueva TA. Applied biochemistry and Microbiology 2005; 3: 261-282. *(in Russian)*
[16] Pijikova GV, Karaseva EV. Agricultural biology 1985; 12: 112-114. *(in Russian)*
[17] Vulettic MV, Sukalovich H. Plant Science 2000; 157: 257-263.
[18] Gofman YuYa, Vaysblay IM. Applied biochemistry and Microbiology 1975; 5: 777-783. *(in Russian)*
[19] Hippeli S, Heiser I.Plant Physiol Biochem 1999; 37: 167-178.
[20] Dumas B, Freyssinet GK. Plant Physiol 1995; 107: 1091-1096.
[21] Davoine C, Le Deunff E, Led N, Avice JC, Billard JP, Dumas B, Huault C. Plant Cell Environ 2001; 24: 1033-1043.
[22] Troshina NB, Maksimov IV, Yarullina LG, Surina OB, CherepanovaEA. Cytology 2004; 11: 1001-1005. *(in Russian)*
[23] Dröge W. Physiol Rev 2002; 82: 47-95.
[24] Passardi F.Phytochemistry 2004; 65: 1879-1893.
[25] Zhao H, Wang Q, Zhao H, Wang J. Colloids and Surfaces B: Biointerfaces 2005; 44: 36-10.
[26] Burkhanova GF, Yarullina LG, Maksimov IV. Plant Physiology 2007; 54: 104 -110. *(in Russian)*
[27] Zhu-Salzman K, Zeng R. Insect Science 2008; 6: 477-481.
[28] Revina TA, Gerasimova NG, Kladnitskaya GV, Chalenko GI, Valueva TA. Applied biochemistry and Microbiology 2008; 1: 101-105. *(in Russian)*
[29] Zhao Y, Botella M, Subramanian L, Ni X, Nielsen S. Plant Physiol 1996; 4: 1299-1306.
[30] Major J, Constabel C. Plant Physiology 2008; 146: 888-903.
[31] Lucena MA, Romero-Alanda R, Mecardo JA. Physiol Plant 2003;18: 422-429.